

Shifted Weighted Annotation Network (SWAN) Analysis: Usage, Background, Installation, and Tutorial

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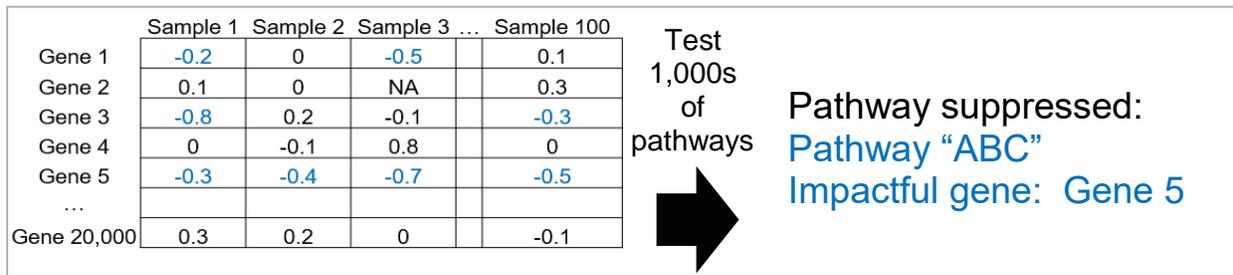
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Purpose of SWAN

SWAN is an analytical tool used to find biological patterns between samples. Its primary purpose is to test for pathway alterations. A typical analysis will include a set of experimental samples compared to control samples, both of which have whole-genome gene information available (such as copy number alterations or RNA changes).

The SWAN App was designed to make interpretation and analysis of copy-number alteration (CNA) data obtained by The Cancer Genome Atlas (TCGA) or custom data accessible to those without a bioinformatics background. The SWAN Shiny App can run on low- or high-powered computers and is intended for ease of use. However, an R package version of SWAN is also available for bioinformaticians which is additionally programmed to enable multi-core analysis on supercomputers and loop across samples.

Since each pathway consists of many genes, the algorithm also prioritizes which genes most impact tumor biology for each set of tumors analyzed. It does that within a single pathway, and also cumulatively adds these effects across all studied pathways to ascertain if a gene can influence many biological pathways. This enables a targeted pursuit of genes and proteins for molecular biology studies.



Data from each pathway can be downloaded per sample for further analysis. This is useful for clustering or survival analyses.

There are two main SWAN programs:

- **Pan-pathway SWAN.** This discovery-based method will test hundreds or thousands of molecular pathways for alterations within your data. It will highlight the most suppressed and most elevated pathways, and which annotations (usually genes) most contributed to this change.
- **Single-pathway SWAN.** Once you discover a pathway of interest for your study, this program expands the data available to you to allow for a deeper-dive into the genes involved in creating the pathway alteration.

Both come in an online App form, which is free for academic users. The pan-pathway version is available as open-source R package functions for bioinformaticians and as a downloadable Shiny App.

Introduction

Origin of SWAN: Copy-Number Alteration Analyses of Human Tumors

To preface the rationale behind the design of SWAN, it is important to note how cancerous tumors have changed their landscape of copy-number from the normal two-copies per gene. Somatic copy-number alterations (CNAs) by definition are changes in the dosage of genes from what a patient has in their normal genome. Most genes are affected by CNAs resulting from entire chromosome arm gains or losses. Because most chromosome arms contain essential genes, homozygous losses are extremely rare, and occur in <1% of gene CNAs. These homozygous deletions are “focal”; that is, they occur on some smaller portion of a chromosome arm. Similarly, many extra copies of a chromosome are detrimental to cell division, so having twice the dosage of a gene is rare, and <5% in any tumor type. These genes are often isolated on “double-minute” chromosomes – broken chromosomes which can replicate and segregate independently of the normal genome, or on extra chromosomal DNA.

Hence, >90% of the gene-level CNAs within a tumor are monoallelic; that is, either the loss of one allele (while one allele remains) or the gain of a single allele (to three alleles total). The scale of this is much higher than single-nucleotide variants / indel mutations in tumors. While most tumors have SNVs in <1% of genes, tumors range from 10-70% of their genome altered by CNAs. Clearly, it is important to understand how these CNAs alter biology in tumors. That is the purpose of SWAN and the Atlas, here.

While epigenetic states can alter the expression of genes on these CNAs, and often does, there are clear trends toward a correlation of RNA expression and even protein expression with CNA changes. For references, please read PMID:[27165746](#) (shows 93% of expressed genes on a CNA heterozygous loss are reduced in expression) and PMID:[27372738](#) (shows 90% correlation of mRNA to protein).

One significant caveat is that CNAs can often be formed through a whole-genome duplication event first, followed by losses and gains. Hence, instead of 2N normal copy number, the “normal” copy number for this duplicated genome cell is 4N. Thus a “heterozygous loss” would be a 2N copy number and a “heterozygous gain” would be 6N in copy number. While more intuitively complex, studies in yeast and plants suggest these duplicated genomes still function similar to normal 2N genomes in terms of pathway flux, enabling a relative determination of suppressed or enhanced pathways across tumors.

SWAN Uses: Not Limited to Cancer

While cancer was the initial reason for developing SWAN, diploid organisms follow evolutionary biology which is highly relevant to SWAN. For example, sibling differences are often due to differences in single gene alleles. While siblings contain similarities, there are usually clear differences as well. These differences are the core selective fodder for Darwinian evolution. While other systems were not involved in the design of SWAN, we did test neurodegeneration RNA data and saw expected neuronal pathways as the top hits. Evaluation of datasets involving organismal aging in mice also showed previously published pathways were hits, along with additional novel hits. Just like other pathway analysis tools, we expect SWAN to help discover novel biology in many model systems. Custom uploads enable users to query any organism or annotation, as long as users can supply pathway set(s) and interaction data to build networks.

The SWAN Algorithm

Concept. Biological data can often contain great levels of noise. To obtain significant differences in highly heterogeneous data, pathway analysis may be used. To further prioritize which genes most drive pathway differences, SWAN build networks within each pathway. Networks are scored based on (1) how many interactions occur within the pathway with individual genes (higher score), (2) if a gene has haploinsufficiency data associated with it (higher score), and (3) the value of the gene within each sample (may be positive, negative, or zero).

Controls. To achieve significance, the observed data must be compared to a control. There are two options for controls: (1) a permuted background control, which performs an in silico randomization of all gene-level data for each network, and (2) a user-supplied control, which should be in the same scale and format as the experimental sample data. In the case of a user supplied control, permuted background networks must still be generated and scored to provide estimates of shifts expected by chance. Controls are the foundation of SWAN, and the reason why many background permutations are recommended for most analyses.

SWAN Inputs

The basics: SWAN requires gene-level data on a consistent scale, such as log₂ intensity values or integer copy numbers (with normal ploidy set at zero). Example data in the correct format can be downloaded using the online App. Columns represent samples, and rows represent genes or other annotations.

The details: SWAN has many input criteria which can aid calculations of specific problems. Each input variable is briefly **described below**:

Main inputs (necessary for all SWAN analyses)

<i>Type of analysis</i>	Select from TCGA demo data or Custom to use your own data
<i>Experiment data file</i>	Tab or comma delimited file containing your gene-level data as rows and samples as columns. Data should be normalized to a -2 to +2 range
<i>Control data file</i>	(Optional) upload similar to experiment data file, for control samples
<i>Pathway set</i>	Choose from pre-installed pathway sets. If needed, custom pathway sets may be uploaded in the “other options” section

Other options (for customizing analysis)

<i>Control permutations</i>	This is the “background” calculation performed to determine what may be found by chance. A value of 200 is recommended for confident results, and 1,000 for publication-quality analysis
<i>Significance threshold</i>	FDR threshold for what is considered as “significant”. Can be set as 1 to include all resulting pathways

<i>P-value setting</i>	Toggle between network-level P-value configuration and gene-level P-value configuration. Gene-level is less restrictive but may risk false positives, whereas network-level is more restrictive and may risk false negatives
<i>Score interactions</i>	SWAN will prioritize genes based on how many proteins within a pathway a gene product interacts with
<i>Haploinsufficiency</i>	SWAN will prioritize genes based on known haploinsufficiency and triploproficiency data from mouse and yeast homologs
<i>Custom gene scores</i>	SWAN will prioritize genes based on this upload. This may be useful to coordinate SWAN analysis with other phenotypic data
<i>Score mutations</i>	Similar to “custom gene scores”, this allows a file to be uploaded containing data marking which genes are mutant. These genes will be prioritized by the multiplication factor also set here
<i>Score RNA</i>	This may be used with or without mutation scoring. It also prioritizes genes based on a custom file upload. Data should be appropriately scaled to the -2 to +2 range. There is an option to add RNA data to the analysis only when consistent with main experimental & control file(s)
<i>Value cutoffs</i>	For data which is not in -2, -1, 0, 1, 2 scale but rather continuous, this is a necessary setting to enable SWAN to mark which genes are changed to monoallelic or higher levels. A common value set for CNAs is +/- 0.2 for monoallelic alterations, and +/- 1.0 for multiple allele alterations.
<i>Pathway size</i>	Some pathway sets may include uninformative small pathways (<10 genes) or uninformative giant pathways (>200 genes) which complicate interpretation of hit genes. The defaults here limit pathways to those enabling downstream analysis, but larger or smaller pathways may be included by changing this threshold
<i>Ignore gene(s)</i>	A common experiment is knocking down or knocking out a gene. In this case, analysis of all pathways containing that gene will be biased by the loss of that gene. To ignore the manipulated gene, enter the gene symbol here. Biological changes which result from loss of the gene will then be analyzed.
<i>Custom files</i>	Custom pathway or interaction files may be uploaded. This enables an analysis of organisms outside of SWAN defaults, or for something other than BioGRID protein-protein interactions to be used in network creation

SWAN Data Groomer

Since SWAN will only run on specifically formatted data, the Data Groomer was released to enable users to automatically adapt existing data for the format SWAN requires.

The Data Groomer can map *.seg files onto genes (and convert genome coordinates depending on how your *.seg file was generated). If using gene-sample pair format, it can log₂ normalize your data, remove genes with zero data, find Gene Symbols from other database identifiers, and scale your data to the -2 to +2 range SWAN is optimized for. If you have DNA, RNA, and protein data for the same samples, simply run the Data Groomer for each data type one at a time.

Instructions can be found at the Data Groomer App webpage:

https://delaney.shinyapps.io/SWAN_Data_Groomer/

Please note that consulting a statistician will almost always be a better option than an automated tool like the Data Groomer.

SWAN Outputs

Pan-pathway SWAN.

You can download some of the spreadsheets which generate the statistical data using these buttons:



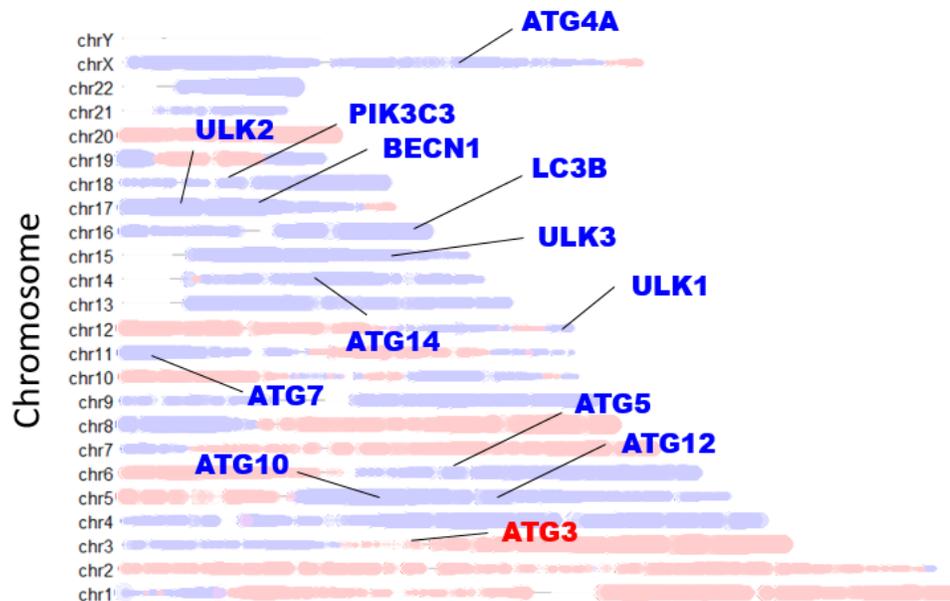
<i>Results summary</i>	Downloads the table containing SWAN pathway analysis
<i>Pathway per sample</i>	Downloads SWAN shifts in tabular format for every sample, enabling hierarchical and other clustering methods and other downstream analyses
<i>Interactome</i>	Downloads a cumulative SWAN shift per gene across all pathways analyzed. The genes with the highest magnitude may alter the most biology across samples, although cumulative magnitude is biased by how many pathways each gene appears in.

Single-pathway SWAN.

Graphical outputs are described below in the Example / Tutorial.

An example of CNAs altering a tumor suppressor pathway

Autophagy gene locations and Ovarian Cancer CNA distribution:



Red indicates CNAs predominantly are gains/amplifications in the OV cohort, blue indicates CNAs are predominantly losses within the cohort. Clearly, most autophagy initiation genes lie on chromosome regions selected for losses, although ATG3 does not.

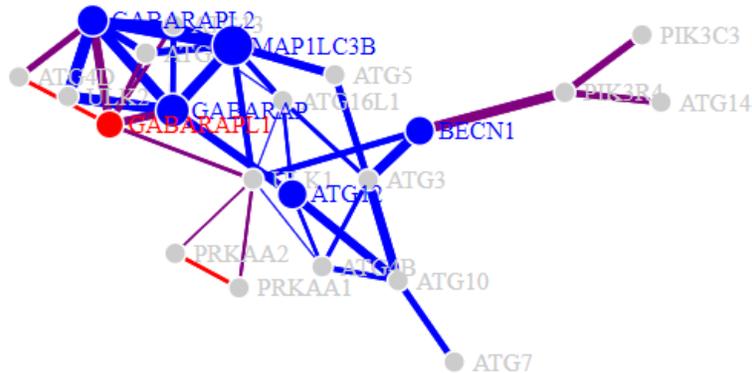
SWAN Single-Pathway App

SWAN Single-Pathway App Overview

Once the Pan-pathway SWAN test has been run, the Single-Pathway App may delve further into the data. This is intended to improve upon the pan-pathway overview in the following ways:

- Data for all pathway genes can be tabulated
- Graphical outputs of the pathway's network can be generated and plotted
- Graphical outputs of each sample's annotations can be plotted
- Pathway genes can be plotted along chromosomes, to identify chromosome arms which may be most important to altering the pathway
- Enables querying of a single pathway of interest, rather than all pathways, if a hypothesis exists
- Can quickly incorporate other data for pathway of interest (RNA, mutation, differing controls or subsets of experimental data)

SWAN Single-Pathway App Example and Outputs
 SWAN Impact Network:



This interactive network highlights which annotations most influenced the overall network score. The color key is as follows, according to sample averages:

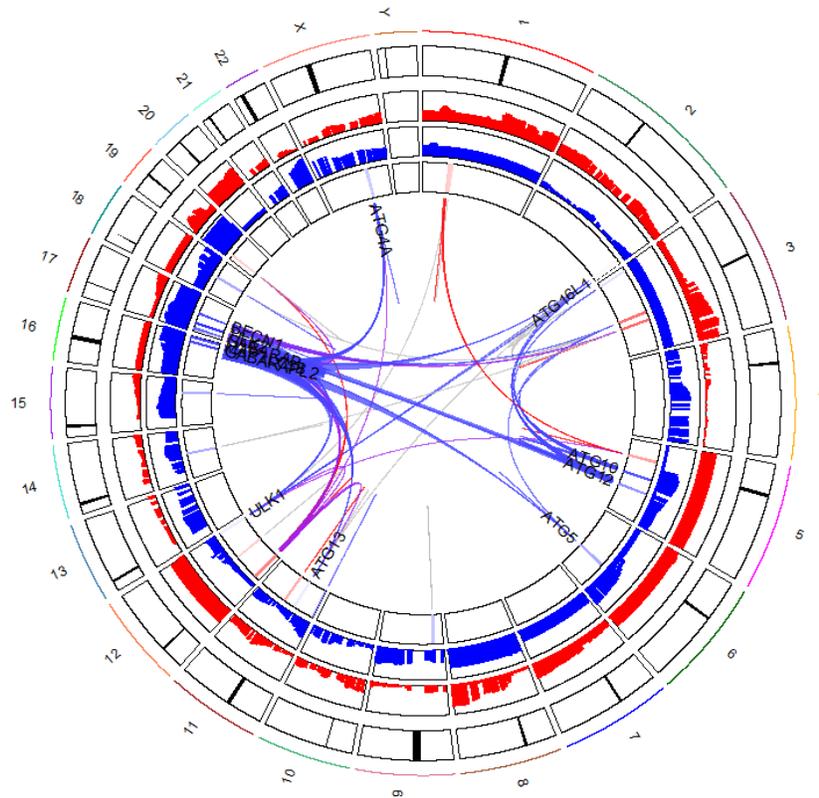
Nodes

- Dark blue** Highest quartile of negatively scored annotations
- Blue** 3rd quartile of negatively scored annotations
- Light blue** 2nd quartile of negatively scored annotations
- Gray** Between light blue and light red scoring levels
- Light red** 2nd quartile of positively scored annotations
- Red** 3rd quartile of positively scored annotations
- Dark red** Highest quartile of positively scored annotations

Edges

- Blue** Both nodes are negatively scored
- Gray** Both nodes are neither negatively nor positively scored
- Red** Both nodes are positively scored
- Purple** Nodes are antagonistic; one is negatively scored, the other is positively scored

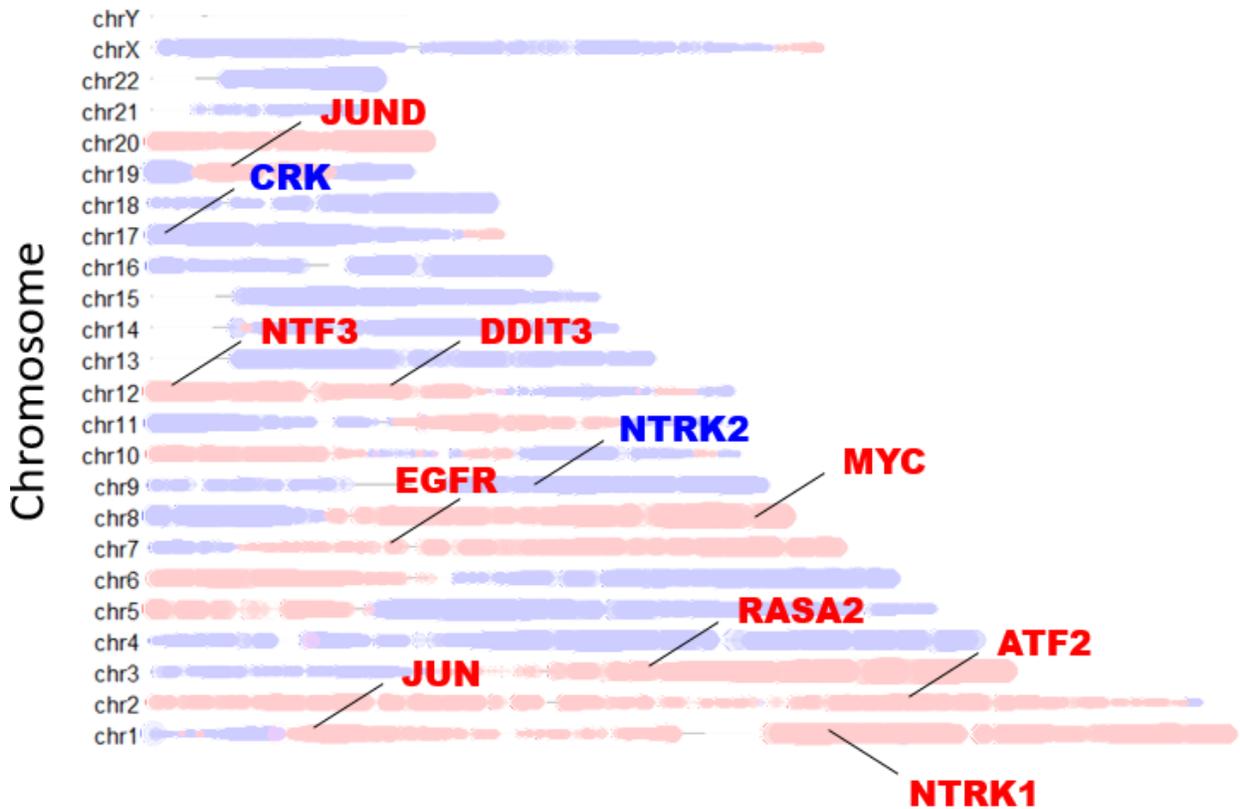
SWAN Circos Network



Each chromosome is labeled as the outside ring, with the inner red and blue rings marking the frequency of **gains** or **losses** on each region of the chromosome, respectively. Connections inside the Circos plot (“ribbons”) represent protein-protein interactions. The thicker the line, the more that gene influenced the SWAN score for the pathway’s network. Gene labels are included if a connected ribbon score is beyond one standard deviation from a zero change value.

An example of CNAs altering an oncogenic pathway

MAPK pathway gene locations and Ovarian Cancer CNA distribution:



While these examples demonstrate the core concept behind SWAN analysis, the reality of tumor genomics and pathway alterations are complex. Most pathways will have some genes enhanced in copy number within a given pathway but also some genes with lower dosage. The balance between these changes are determined and used as an estimate for changes in pathway flux. In addition, pathways can be “dysregulated”: that is, some genes are amplified and some are deleted, resulting in altered pathway function. In this case, the score for the pathway is not statistically significant despite a change in biological effect, since neither hypothesis for simple pathway activation or inactivation are upheld by the data.

In addition to the chromosomal data incorporated from individual tumors, pathway networks are built from protein-protein interactions originating from the BioGRID database (<https://thebiogrid.org/>). Genes encoding proteins which are highly interactive within the pathway’s network will be scored as more “influential”; that is, since they interact with many genes within the pathway, each interaction is scored individually as part of the pathway’s overall scores. Since a gene with many interactions will have many contributions to the score, their influence in the network is larger than a gene without interactions.

Finally, these pathway network scores layer in one more biologically relevant datum. We incorporate a multiplier for a gene if its homologue is known to be haploinsufficient in yeast or mice. This is because most of the genetics changes in CNAs in tumors are single allele changes, thus those genes which influence function with a single allele change are also scored higher.

These calculations in SWAN are put forth in more detail in the original publication (pending), along with quality control specifications for how we settled on these factors within the pathway calculation. In short,

these help prioritize known tumor suppressors and oncogenes, giving a higher likelihood that other genes the algorithm prioritizes will also be tumor suppressors or oncogenes.

SWAN improves on Dr. Delaney's previous HAPTRIG algorithm with the addition of the following changes:

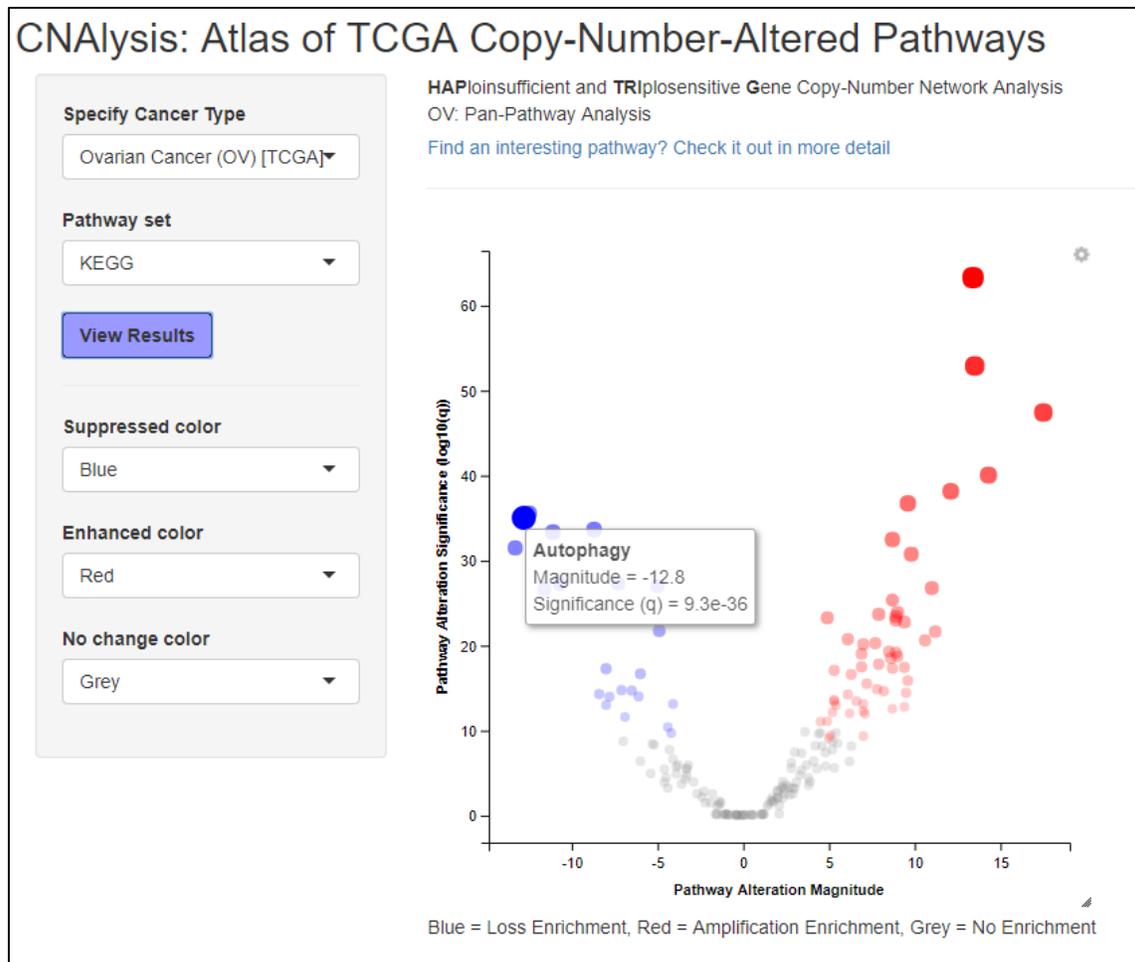
- Development of a Shiny App interface to test a pathway set, rather than a single queried pathway
- Comparison of a control permuted background to another control permuted background to establish a genuine network topology normalization and improve true positives
- Use of an iterative majority vote method to determine pathway suppression or enhancement hypothesis formation, thereby stabilizing p values and reducing necessary randomizations to achieve stable results
- Inclusion of pan-pathway interactome testing to find highest-impact annotations (genes) which influence many suppressed or enhanced pathways
- Ability to test experimental samples to control samples, rather than only experimental samples to permuted background control
- Ability to input a user-designed pathway set
- Ability to input a user-designed scoring matrix to prioritize genes (eg, known causal genes in phenotype data sets)
- Ability to overlay other data, such as RNA and/or mutation, to perform an integrative analysis
- Use of lapply and matrix manipulations to speed calculations 100 fold
- Parallelization options for bioinformatic use on multi-core computers
- Extensive testing across the TCGA to determine optimal cutoffs
- Inclusion of companion functions and Apps to groom the data for input into SWAN

SWAN Pan-Pathway App

Examination of Ovarian Cancer: An example / tutorial

Volcano plot of altered CNA pathways

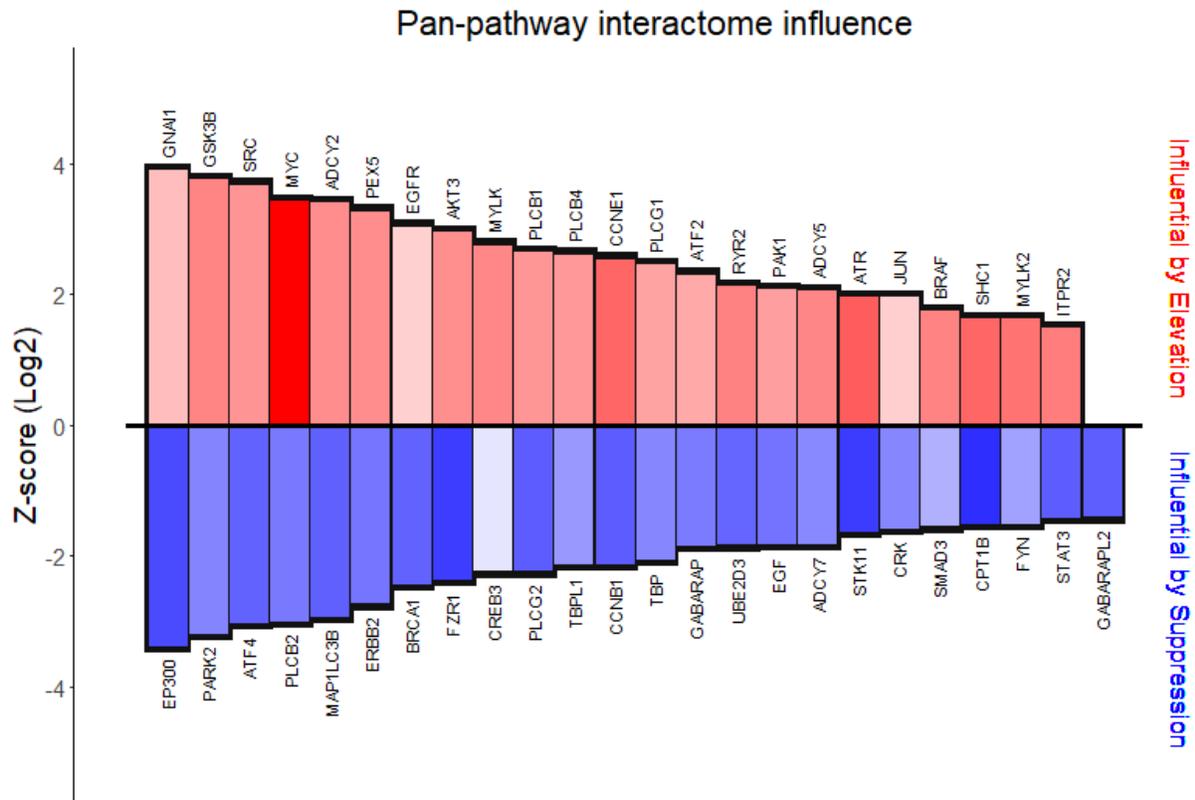
When we first were building this algorithm, we used the Autophagy pathway as an example, since it was the strongest suppressed CNA pathway in serous ovarian cancer. Autophagy is a process which is normally regulated through changes in flux. It is a cellular recycling pathway, so higher flux means more molecules are recycled by autophagy, and lower flux means the cell is unable to completely recycle a normal amount of molecules. Select “Ovarian Cancer (OV)” and “KEGG” and click “View Results” to bring up this screen:



Hover the mouse over the data points to bring up a small window describing the magnitude of suppression and the FDR q value of significance. The magnitude describes the percent shift from the result expected from permuting the data 10,000 times and generating pathway-level network scores for each permutation. It is the shift from the minimum network score as calculated for the hypothesis that the pathway is haploinsufficient. Triploproficient pathway magnitude changes are similarly calculated as the shift from the maximum network score from 10,000 permuted data networks. The significance is yielded by first calculating the median Wilcoxon rank-sum p value from the actual tumor networks relative to the permuted tumor networks and then using a Benjamini Hochberg FDR correction for multiple hypothesis testing. Bonferroni testing was also used, and can be seen in the downloadable pathway summary.

Pan-pathway summary of the most influential genes

Some genes will influence the pathway scores across many different pathways. Since many pathways are affected, these may be more biologically relevant tumor suppressor and oncogenes to target for a particular cancer type. Sometimes these changes are simple to interpret, such as the amplification of MYC in many ovarian cancer tumors:

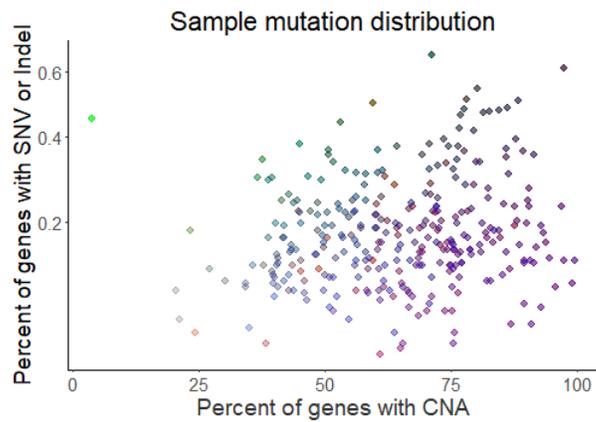
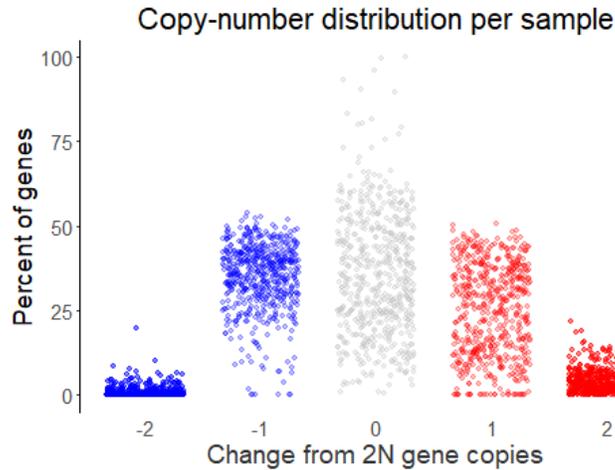


High transparency indicates influence originates from interacting genes

Since MYC is overexpressed in many samples and affects many pathways, it is highlighted in deep red in the above bar graph. However, some genes may also have a strong influence on a pathway if other genes which regulate the given gene are consistently upregulated across tumors, even if that particular gene itself is not always upregulated (this is one advantage of network analysis). For example, in ovarian cancer, many genes which interact with EGFR are upregulated in a variety of pathways, leading to EGFR appearing on this graph, but in a more transparent shade of red. This may mean that pathway inhibition of EGFR-related pathways may still reduce proliferation in ovarian cancer, even if inhibition of EGFR itself may not.

Mutation Plots (TCGA only)

The next two graphs simply show the distribution of CNAs and mutations (as defined by single-nucleotide variants and short indels). Each "sample", represented by a single dot, refers to a single primary tumor from an individual patient. The coloring on the mutation plot shows a gradient: green for unusually high SNV/indels, blue for more loss CNAs than gain CNAs, red for more gain CNAs than loss CNAs, and purple for CNAs which are equally losses and gains within the sample.



Downloadable Data

You can download some of the spreadsheets which generate the statistical data using these buttons:

Download Results Summary

Download Pathway Data Per Sample

Download Pan-Pathway Interactome Influence Data

The “Results Summary” will download the same information as in the displayed table in a single spreadsheet.

Descriptions of the Results Summary header are as follows:

Pathway: Name of the pathway analyzed by SWAN

Result: “Haploinsufficient” means that statistical significance was reached for the hypothesis that this pathway is suppressed by CNA deletions. “Triploproficient” means that statistical significance was reached for the hypothesis that this

pathway is suppressed by CNA gains. “No Selection” means that statistical significance was not reached for either hypothesis.

Model Avg: This is the average of two scores: 1) The shift of the data from permuted background scores, for the “Haploinsufficient” hypothesis, and 2) The shift of the data from permuted background scores, for the “Triploproficient” hypothesis. It is a magnitude estimate of the shift from control data, of arbitrary units.

Wilcoxon p: This is a Wilcoxon signed-rank statistical test with a nominal p value. It tests two groups: the tumor SWAN pathway scores, and the control (in this case, a randomly permuted gene background based on the distribution of gene-level CNAs in each individual tumor).

More accurately, this p-value is a median p-value from the 10,000x(number of samples) control networks calculated for each individual tumor for the whole set of tumors in a paired signed-rank test. Even more accurately, this is the medians of medians; groups of 25 control permuted networks were generated, and then (10,000 / 25 = 400 medians) were taken. These extra median steps were taken to enable a more reliable, stable p-value, which is less dependent on the number of control networks calculated.

q Bonferroni: A Bonferroni multiple-hypothesis-testing correction of the Wilcoxon p value, for the number of pathways within the pathway group.

FDR Benjamini Hochberg: A Benjamini Hochberg multiple-hypothesis-testing FDR correction of the Wilcoxon p value, for the number of pathways within the pathway group.

Std Dev of Model Score: This shows the standard deviation of the “Model Avg” score between tumors in the data set.

Low Gene (1:5): These are the genes within this pathway’s network which contributed to a negative (haploinsufficient) scoring of the network, ranked from the lowest score to the next four lowest scores. These genes are meant to be interpreted only for pathways marked as “Haploinsufficient” in the statistical call.

Low Gene Score (1:5): These are the scores associated with the Low Genes (1:5), in arbitrary units. Negative values show network suppression by deletion events, positive scores may be found if there are not enough suppressed genes within the pathway for this tumor type.

High Gene (1:5): These are the genes within this pathway’s network which contributed to a positive (triploproficient) scoring of the network, ranked from the highest score to the next four highest scores. These genes are meant to be interpreted only for pathways marked as “Triploproficient” in the statistical call.

High Gene Score (1:5): These are the scores associated with the High Genes (1:5), in arbitrary units. Positive values show network enhancement by gain/amplification events,

negative scores may be found if there are not enough enhanced genes within the pathway for this tumor type.

The button “Pathway Data Per Sample” will download normalized pathway shift (from control) data for each tumor in the data set. The header is the tumor sample name.

The button “Pan-Pathway Interactome Influence Data” will download every gene analyzed for all pathways and their cumulative score contribution to all pathways (eg, an oncogene will have a strongly positive score, and a tumor suppressor will have a strongly negative score, as they will affect multiple biological pathways).

Descriptions of the Interactome header are as follows:

nodeScores-

SignificanceCorrected This is the cumulative SWAN score of the indicated gene, summed up for all pathways which involve this gene. The magnitude is further multiplied by the log10 value of the FDR q value of the pathway, for all pathways this gene is involved in. These are not corrected for the number of pathways this gene is involved in; the interpretation of a high magnitude is that the gene is involved in many pathways, and highly suppressing biological changes (if negative) or highly enhancing biological changes (if positive).

z_scores_sig This is the number of standard deviations away from the mean “nodeScores-SignificanceCorrected” value.

nodeScoresRaw This is the same as “nodeScoresSignificanceCorrected”, but without any correction for the significance of the pathways the gene is involved in.

z_scores_raw This is the number of standard deviations away from the mean “z_scores_raw” value.

Quantitative Table Output

The last table is a sortable version of the SWAN output information. You can click the double gray arrows to sort by the clicked column, either by ascending or descending. The header is the same as the “Results Summary” header described above.

Show entries

Search:

Pathway	Result	Model Avg	Wilcoxon p	q Bonferroni	FDR Benjamini Hochberg	Std Dev of Model Score	LowGene1
Arginine and proline metabolism	Haploinsufficient	-13.310811	2.04e-33	3.81e-31	3.18e-32	6.713	SAT1
Autophagy	Haploinsufficient	-12.788349	3.98e-37	7.44e-35	9.30e-36	5.813	MAP1LC3B
Fatty acid degradation	Haploinsufficient	-12.498726	1.10e-37	2.06e-35	2.94e-36	5.519	CPT1B

Installation

The web application does not require any installation. However, if you routinely use SWAN, we would appreciate it if you download the software to help reduce our server costs.

All Shiny Apps, including SWAN, require that you first install to your computer these free programs:

R (<https://www.r-project.org/>)

RStudio (<https://www.rstudio.com/products/rstudio/download/>)

Choose 64-bit installations if you are unsure.

Pan-pathway SWAN installation

Installation of the Pan-pathway SWAN App enables deeper background permutations which increase the pathway suppression or elevation call confidence.

1. Download the SWAN_PanPathway.zip file from GitHub (link). Unzip the folder.
2. Close all other open applications
3. Configure RStudio to run this App by installing packages. These are small files that R uses to execute specialized commands, like those in this App.
4. To do this, open RStudio and enter after the ">" in the lower left-most corner:

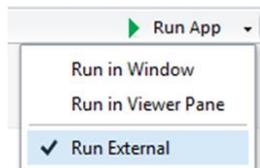
```
install.packages(c("shiny", "data.table", "dplyr", "ggplot2", "ggvis",  
"shinyBS", "shinyjs", "DT"))
```

It should look like this:



The screenshot shows the RStudio interface with a terminal window open. The terminal prompt is ">" and the command entered is `install.packages(c("shiny", "data.table", "dplyr", "ggplot2", "ggvis", "shinyBS", "shinyjs", "DT"))`. The terminal window has tabs for "Console", "Terminal", and "Jobs".

5. Press enter to install packages. It will take some time to install all the packages, click "yes" when prompted. Note **any red text** which appears: this may indicate you need to install additional packages or software on your particular computer.
6. Quit RStudio and restart your computer prior to first use of the Shiny App.
7. Navigate to the main directory you unzipped the files. You should see two .R files: "ui.R" and "server.R". Open either of them in RStudio.
8. Find the green arrow next to "Run App". Click the small black arrow adjacent to "App" and click "Run External". See screenshot below:



9. You are ready to go! Click the green arrow once and SWAN will run in your default browser.

R Package SWAN Installation

For advanced users only. Please download from GitHub.

License

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Any usage or distribution of the underlying SWAN package code is subject to GNU General Public License V3 (GPLv3) - <https://www.gnu.org/licenses/gpl-3.0.en.html>

Any usage or distribution of the SWAN Shiny App code is subject to the MUSC License:

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